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PRODUCTION AND ANALYSIS OF THE BIOPOLYMER CHITOSAN FROM *MUCOR ROUXII*

BY

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To determine the potential for the production of chitosan with desired physiochemical properties from a fungal source, growth studies were conducted using Mucor rouxii. Growth of the organism under a variety of conditions was studied to determine the effects on polymer molecular weight distributions and biomass production. Modifications of processing protocols were also evaluated to examine the effects on yields of chitosan extracted from the fungal cell wall as well as molecular weight distribution. This represents the first such study where these factors were correlated to the yield and molecular weight distribution of chitosan. Of the growth parameters evaluated, length of incubation, culture volume, source of inorganic salt in defined medium, and medium component concentration in complex medium had an effect on biomass and MW distributions. Processing parameters affecting the amount of chitosan extracted were the type and strength of acid and the homogenization of cell wall material prior to refluxing. Some of the factors which had no influence on the MW distribution of chitosan included glucose concentration and addition

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19. (cont'd) of trace elements to complex medium.

The average molecular weight of chitosan extracted from the cell wall of _M. rouxii_ can be controlled to some extent, with weight average molecular weight ranging from 200 K to 1.2 million and a dispersity around 7. The low yields of chitosan from _M. rouxii_ (5% to 10% of total dry weight of biomass) are comparable to that of commercial chitosan production yield from shellfish waste (approximately 10% of raw material). _M. rouxii_ may become a viable source of chitosan in the future, especially if processing procedures can be made more efficient. Sufficient quantities of chitosan have been isolated for research purposes, however, alternative approaches are under evaluation for large-scale production.

Overall, weight average molecular weights of chitosan varied up to 8-fold in studies relating to fungal age, while up to 2-fold changes in molecular weight were effected by pH, medium type, and culture vessel size.

PREFACE

This report contains the results of a study of the production of chitosan from a fungal source, Mucor rouxii. This study was funded under the U. S. Army Natick Research, Development and Engineering Center (Natick RD&E Center) Program Element 61101A, Project No. 1L161101A91A, Task No. 07, Work Accession No. 144.

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INTRODUCTION

Chitosan, a cationic polymer consisting of β-1,4 linked 2-amino-2-deoxy-D-glucose, is rarely found in nature. The primary occurrence is as a cell wall component of some fungi, particularly the Zygomycetes, in addition to chitin. Chitosan has been identified in the genus *Mucor* (2) and *Phycomyces* (7). Commercially, chitosan is derived from chitin, which is obtained from crustacea wastes. In this process, a strong alkali digestion is required to deacetylate chitin to produce chitosan (Fig. 1), and variability in source material leads to variable physicochemical characteristics. The use of a fungus as a source of chitosan avoids the strong alkali digestion and there is, therefore, less concern about possible batch to batch inconsistencies and in effects on molecular weight (MW) distribution of the final product. The use of a fungus also has the potential to provide closer control over the physicochemical properties by controlling fermentation and processing parameters. The potential also exists for genetic manipulation using the fungal system.

Background

Chitosan was first identified in 1859 by Rouget (15) from chitin boiled in caustic potassium hydroxide and solubilized in dilute organic acids. Chitosan was first found in nature in 1954 by Kreger (7) in the cell walls and sporangiophores of *Phycomyces blakesleeanus*, and subsequently in *Mucor rouxii* by Bartnicki-Garcia and Nickerson (2) in 1962. By 1978, Fenton, *et al*. (5) found that each family of the Zygomycetes contained chitosan in their cell walls.

The degree of acetylation of the glucosamine polymer determines whether it is chitin or chitosan by influencing the solubility properties. Chitosan produced from shellfish chitin ranges from near 0% to 50% acetylation (6), and cell wall chitosan isolated from *M. rouxii* has been reported to contain a 5% to 10% acetyl content (18). The chitosan content of the cell wall is reported to be 33% for the mycelia, and 28% for yeast-like forms (2). The presence of CO_2 during incubation has been demonstrated to affect the morphology in *M. rouxii*. Incubation in nitrogen gas or air yields the typical filamentous mycelia, while budding yeast-forms are formed under CO_2 (3).

Previous work concerning chitosan from fungal sources has not dealt with the aspect of MW distributions of the polymer product. White's choice of extraction parameters was based solely on yield of chitosan (18). Molecular weight distributions have been determined for chitosan produced from the commercial conversion of shellfish waste. A commercial chitosan has been

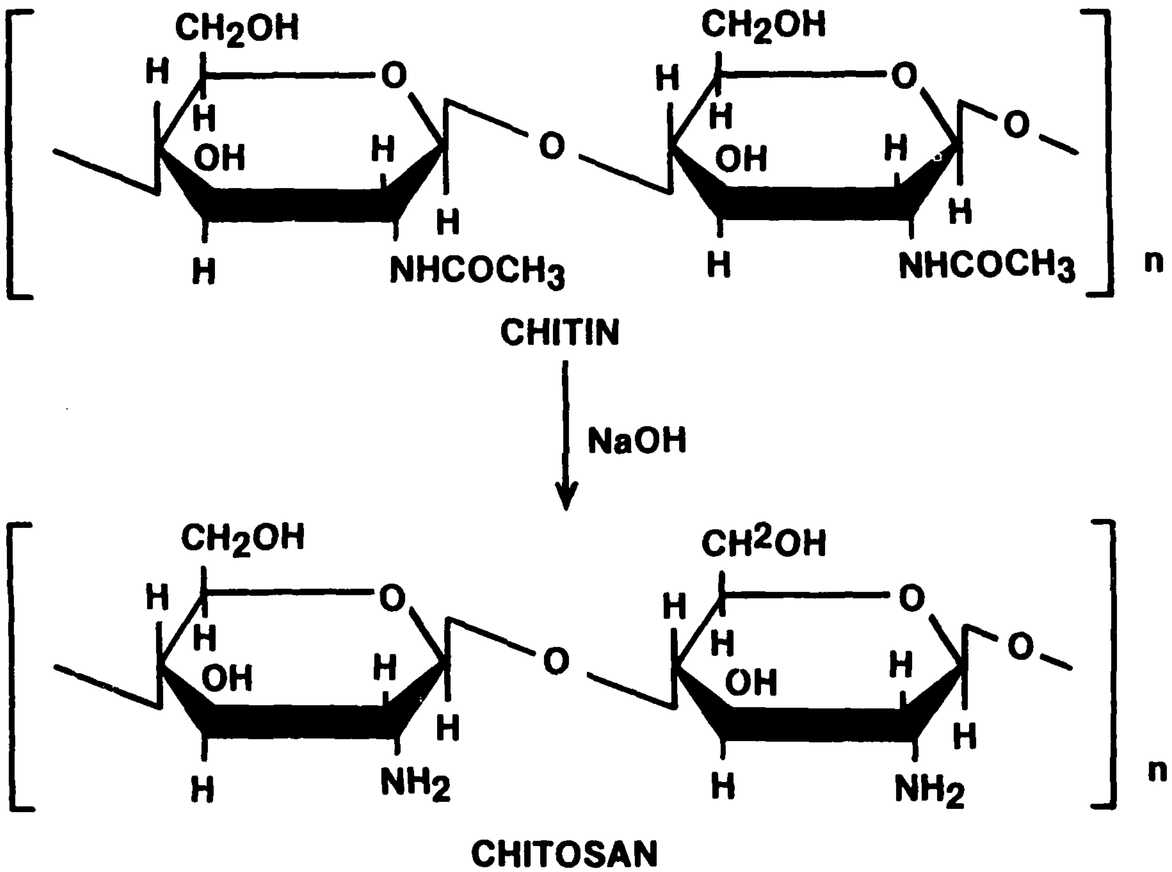

FIGURE 1. STRUCTURE AND CONVERSION OF CHITIN TO CHITOSAN

reported to have a weight average MW of 2.06 million as determined by High Performance Liquid Chromatography (HPLC) using dextran standards for calibration (19).

The list of potential and existing applications for chitosan are extensive, including adhesives, food processing, paper and textile additives, wound healing accelerants, and waste water treatment (8).

Objective

The objective of this study was to characterize the yields and MW distribution of chitosan produced and extracted from the mycelial cell wall of the fungus *M. rouxii*.

MATERIALS AND METHODS

Microorganism

Mucor rouxii (ATCC 24905) was maintained on yeast extract-peptone-glucose (YPG) agar slants, pH 5.0 and stored at 4°C. YPG medium consists of the following ingredients per liter: yeast extract, 3.0 g (Difco Laboratories, Detroit, MI); peptone, 10.0 g (Difco); Bacto agar, 15.0 g (Difco); and glucose, 20.0 g (J. T. Baker Chemical Co., Philipsburg, NJ). For long term storage, a spore stock was prepared by washing three day old growth on YPG plates with sterile distilled water containing 0.05 g/L Tween(R) 80 (Atlas Powder Co., Wilmington, DE). The spores were centrifuged 3000 x g for 10 min. and the supernatant discarded. The spores were then washed twice with sterile distilled water followed by one wash with mineral salts solution consisting of the following per liter: KH_2PO_4, 0.7 g; K_2HPO_4, 0.7 g; $MgSO_4 \cdot 7H_2O$, 0.7 g; NH_4NO_3, 1.0 g; NaCl, 0.005 g; $FeSO_4 \cdot 7H_2O$, 0.002 g; $ZnSO_4 \cdot 7H_2O$, 0.002 g; $MnSO_4 \cdot H_2O$, 0.002 g (Fischer Scientific Co., Fairlawn, NJ). The spores were then suspended in the mineral salts solution, and titered with a hemocytometer. A 24% sucrose or 20% skim milk solution as an osmotic stabilizer was added to the spore suspension 1:1 (v/v) before rapidly freezing in -70°C acetone-dry ice and lyophilization. The lyophilized spores were stored at -18°C.

Media

Three different culture media were used for evaluation of biomass production and MW distribution of chitosan. Two complex media were evaluated, BG and YPG. BG consists of the following ingredients per liter: nutrient broth, 8.0 g; yeast extract, 0.1 g (Difco); KCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.278 mg; $MnCl_2 \cdot 4H_2O$, 0.002 g; glucose, 5.0 g (Fischer). YPG components were the same as above without agar. A defined medium, TVB, was

evaluated and contained the following per liter: $(NH_4)_2SO_4$, 1.4 g; KH_2PO_4, 2.0 g; $CaCl_2$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; molybdic acid 85%, 0.01 g; glucose 20.0 g; trace metal solution (see below), 1.0 mL. Citrate buffer, pH 4.4 at 0.05 M, consisting of citric acid anhydrous, 5.38 g/L (Baker) sodium citrate, 6.47 g/L (Fischer) was used if buffering was desired. The trace metal solution contained the following: 495 mL distilled water, 5.0 mL concentrated HCl (12 M) (Baker), $FeSO_4 \cdot 7H_2O$, 2.5 g; $MnSO_4 \cdot H_2O$, 0.98 g; $ZnCl_2$, 0.83 g; $CoCl_2 \cdot 6H_2O$, 1.0 g (Fischer). For all liquid media, glucose was prepared separately and added aseptically after autoclaving. If pH control was necessary, addition of 1N NaOH to the culture was made twice daily. The pH measurements were performed with a Model 130 Corning pH meter (Corning Glass Works, Corning, NY).

Culture Conditions

Fungal growth was studied in batch and continuous cultures. Small-scale batches involved shake cultures of 750 mL in 2800 mL Fernbach vessels, or 100 mL in 250 mL DeLong flasks. Inoculation of 750 mL BG and YPG cultures was from 72-hour-old BG and YPG agar plates, respectively, by homogenizing two 16 mm agar plugs in approximately 50 mL sterile distilled water. The 750 mL TVB cultures were inoculated from 72 hour YPG agar plates. The homogenate was added to the Fernbach vessel, incubated at 25°C and agitated at 125 rpm on an Environ-Shaker Model 3597 (Lab-Line Instruments, Inc., Melrose Park, IL). The 100 mL shake cultures were prepared by the addition of 0.8 mL of the spore suspension (1×10^8 spores). The suspension was stored in mineral salts solution at 4°C.

Large-scale batch cultures consisted of 10 liters of culture medium in a 14 liter fermentor vessel on a Magnaferm MA-114 (New Brunswick Scientific Co., Inc., Edison, NJ). A 24 hour 1000 mL shake flask culture was prepared with the YPG plug inoculum, and added to 9 liters of the same medium as a 10% (v/v) inoculum. Scale-up using the spore suspension began by adding 0.8 mL of the suspension to 100 mL of medium, and incubating for 24 hours at 25°C. After homogenizing, this was added as a 10% (v/v) inoculum to 900 mL. The same steps were repeated for the 1 liter culture, which was the 10% (v/v) inoculum for the 10 liter culture. The 10 liter fermentor was adjusted to 24°C, 1200 rpm agitation rate, and aerated at 5 liters/min sterile air. Prior to autoclaving, 1 mL of SAG 100 dimethylpolysiloxane antifoam (Union Carbide, Corp., Sistersville, W. Va.) was added.

The continuous culture was started as a large-scale batch culture. Agitation, aeration, and temperature were as described for the 10 liter batch cultures. When the culture reached active biomass production, as determined from the 10 liter batch time study data, fresh medium was pumped into the vessel at 0.5, 1.0 or 2.0 liter(s) per hour. Harvesting of mycelium was accomplished by pumping approximately 125 mL of culture medium

from the 10 liter vessel every 15 minutes.

Processing

Mycelia were collected by vacuum filtration and washed with distilled water. An aliquot was dried overnight in preweighed aluminum pans at 100°C to obtain biomass dry weight.

The progress of the extraction was monitored by light microscopy of cell wall material stained with Lugol's stain. The stain contains iodine, 4.0 g (Mallinckrodt Chemical Works, New York, NY), and KI, 6.0 g (Fischer) to a final volume of 100 mL. The cell wall material appears pink to violet, debris from the cytoplasm yellow, and intact mycelia reddish brown (18).

Extraction of chitosan from the fungal cell wall was based on the procedure developed by White, et al. (18) with some modifications. These changes included the elimination of lyophilization of NaOH-treated (cell wall) material, homogenization of the cell wall material with a Waring blender in 2% acetic acid prior to refluxing, and an increase in cell wall-acid ratio to 1:≥100. These modifications of White's method were examined in relation to their effect on MW of chitosan and yield of the product. Other changes were evaluated visually by using Lugol's stain. Modifications included pretreatment of mycelium by soaking or boiling in water; placement of mycelium in -70°C acetone dry ice bath to disrupt the cell wall; and sonification (Cell Disrupter 350, Branson Sonic Power Co., Danbury, CT) of mycelium homogenate prior to NaOH treatment, also for cell wall disruption.

Derivatization of Chitosan

The derivatization of chitosan was based on a method used to produce a water-soluble form of chitin (12), with some modifications. The material to be derivatized was chitosan produced by *M. rouxii* incubated in defined buffered medium for 65 hours. Changes in the procedure included increasing the amount of methanesulfonic acid from 14 mL/2 grams chitin to 25 mL/0.5 grams chitosan, and elimination of the *in vacuo* concentration of derivatized chitosan solution. Phosphorus pentoxide was added at 2.0 molar equivalents of chitosan.

Analytical Methods

A 3 mL to 5 mL aliquot of the culture medium filtrate was retained for glucose determination. The filtered culture medium was injected, a volume of 25 μL, into a YSI 23A Glucose Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) for glucose level determination. Determination of chitosan MW and dispersity was performed on a Waters 150-C ALC/GPC Gel Permeation Chromatography (Waters Chromatography Div., Millipore Corp., Milford, MA). The system was calibrated using polysaccharide

standards ranging in MW between 12,000 and 853,000 daltons (Polymer Laboratories Ltd., Church Stretton, UK). The standards were run through three Bio-Gel (Bio-Rad Laboratories, Richmond, CA) columns; a TSK-60, separating in the 40,000 to 8,000,000 dalton range, followed by two TSK-50 columns, effective from 4000 to 800,000 daltons. A calibration curve was generated correlating weight average MW, number average MW, and dispersity with retention time on the columns. The instrument automatically interpolated the curve and calculated the sample MW distribution and dispersity, including MW distributions greater than 853 K. Standards and samples were solubilized at 0.1% (w/v) in an aqueous solvent of sodium acetate, 0.1 M; acetic acid, 2% (v/v); and sodium azide, 0.05% (w/v). The instrument was adjusted to 1.0 mL/min flow rate, and a 40 minute run time. Sample injection volumes were 200 μL to 300 μL.

Practical grade commercial chitosan derived by the chemical conversion from crab shell chitin was obtained from Sigma Chemical Co. (St. Louis, MO). This material was run on the GPC in the above aqueous solvent at 0.1% (w/v) concentration. The commercial chitosan and the 853 K pullulan (linear α-D-glucan) standard were periodically run as calibration standards to monitor the performance of the GPC.

Chitosan samples were clarified by one of two methods, centrifugation at 3000 rpm or filtration using one of the following systems: a Swinnex filter (0.45 μm); a Millex-HV filter (0.45 μm) (Millipore Corp., Bedford, MA); or a 0.5 μm sintered stainless steel GPC filter (Waters).

Three methods were evaluated for determining the degree of acetylation of chitosan, infrared (IR) spectrophotometry (16), first derivative of UV absorbance spectrum (10), and titration. To determine the percent acetylation by titration, 0.05 g of chitosan was dissolved in 5 mL of 0.1 N hydrochloric acid (standardized with 0.1 N sodium carbonate), with shaking on a wrist action shaker (Burrell Corp., Pittsburgh, PA) overnight. Ten mL of water purified by reverse osmosis was added to reduce the viscosity of the solution. The chitosan solution was then titrated against 0.1 N NaOH on an Automatic Titration System (ABU80 Autoburette, TTT80 Titrator, and PHM82 Standard pH Meter, Radiometer America, Cleveland, OH). The determination of the percent deacetylation of chitosan used the following calculations:

Moles total hydrogen ion (H^+): (normality HCl/L x 5 mL)
Moles free hydrogen ion: volume titrant x (normality NaOH/L)
Moles protonated amine: moles total H^+ - moles free H^+
Theoretical moles protonated amine: 2.81×10^{-4} M (100% deacetylation)
Percent deacetylation: (moles protonated amine/2.81×10^{-4}) x 100

Variability in MW distribution within and between samples

was evaluated due to the high dispersity (weight average MW divided by the number average MW) of chitosan; around 7. The mean weight average MW and standard deviation were calculated by analyzing multiple aliquots from the same sample by GPC. The variability between samples cultured under identical conditions was also determined by this method.

RESULTS

Complex Media

Initially, the two complex media BG and YPG were compared in 750 mL shake cultures (Fig. 2). The weight average MW distribution for BG was a maximum of 643 K, while YPG reached 539 K, both after three days incubation. The biomass achieved in BG was relatively low, a maximum 3.9 g/L after three days compared to 10.3 g/L for YPG (Fig. 3). Each study used a plug inoculum with an initial sampling time of 16 hours.

A second study involving 750 mL YPG shake cultures was carried out to determine more accurately the effect of length of incubation on weight average MW distribution and yield (YPG (2) in Fig. 2). Cultures were processed every 24 hours for a period of seven days, with an additional sample at 10 days (Fig. 2). The maximum weight average MW was 769 K after one day. Weight average MW continued to decline reaching 200 K to 300 K after 6 days. Biomass increased up to 72 hours before stabilizing between 9 to 11 g/L (Fig. 3).

In 10 liter batch cultures, MW increased rapidly, reaching maximum weight average MW values of approximately 1 million to 1.1 million by two days for plug and spore inoculated cultures (Fig. 4). There was a decline in weight average MW during the three- to seven-day period. While biomass in the plug-inoculated cultures leveled off after two days, cultures inoculated with spores declined steadily from 10.3 g/L at three days to 5.0 g/L at seven days (Fig. 5). Although maximum levels were similar, the 10 liter YPG batches show a more rapid rate of biomass production than the 750 mL cultures (10.4 g/L at 30 hours versus 10.1 g/L by three days).

Defined Medium

The defined medium TVB was studied over a seven-day period in 750 mL batch cultures (Fig. 6). Samples were taken every 24 hours to observe MW distributions and biomass production. In general, MW distributions in the 750 mL shake cultures were significantly higher than those of the complex media. A maximum value of 1442 K occurred at five days, later than peak values in the YPG complex medium. The amount of biomass produced in the defined medium increased rapidly during the first three days, and stabilized at 6.2 g/L to 7.2 g/L (Fig. 7). Although the time in

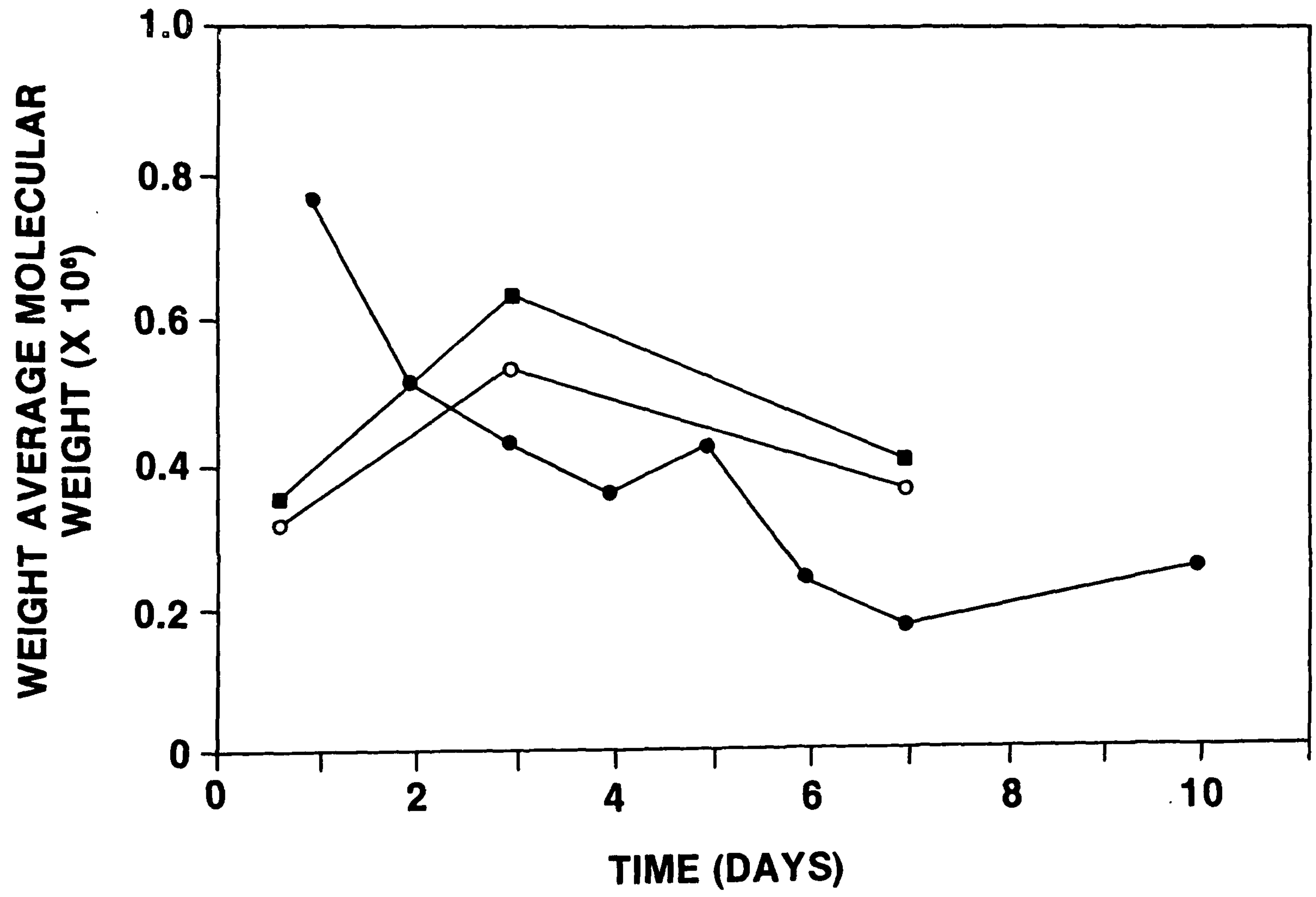

FIGURE 2. WEIGHT AVERAGE MOLECULAR WEIGHT OF CHITOSAN VS. TIME IN COMPLEX MEDIA, 750 ml. YPG (1) (●), YPG (2) (○), BG (■)

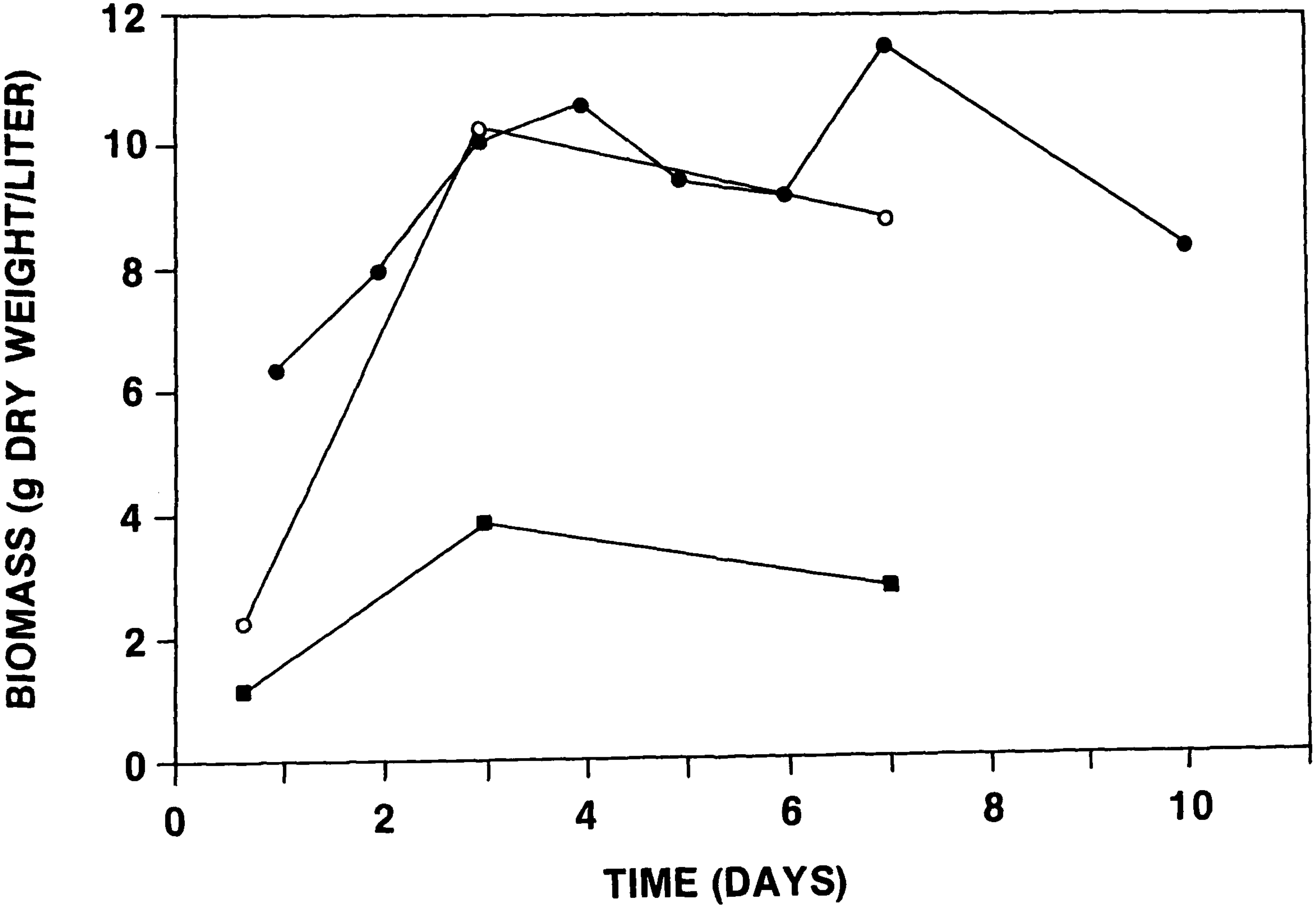

FIGURE 3. EFFECT OF TIME ON BIOMASS PRODUCTION IN COMPLEX MEDIA, 750 ml. YPG (l) (•), YPG (2) (○), BG (■)

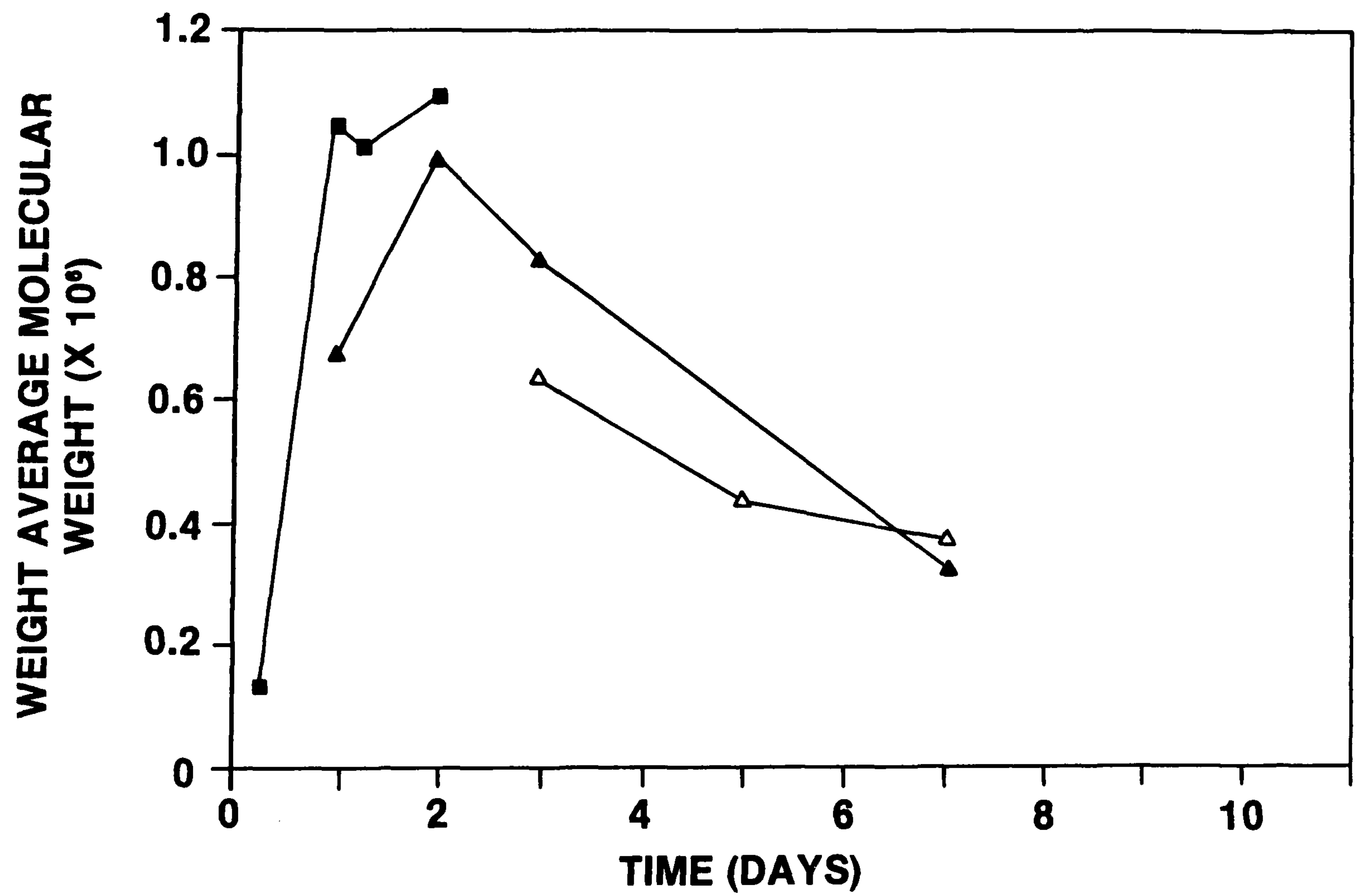

FIGURE 4. WEIGHT AVERAGE MOLECULAR WEIGHT OF CHITOSAN VS. TIME IN COMPLEX MEDIUM, 10 L YPG (1) (■), YPG (2) (▲), PLUG INOCULUM, YPG SPORE INOCULUM (△)

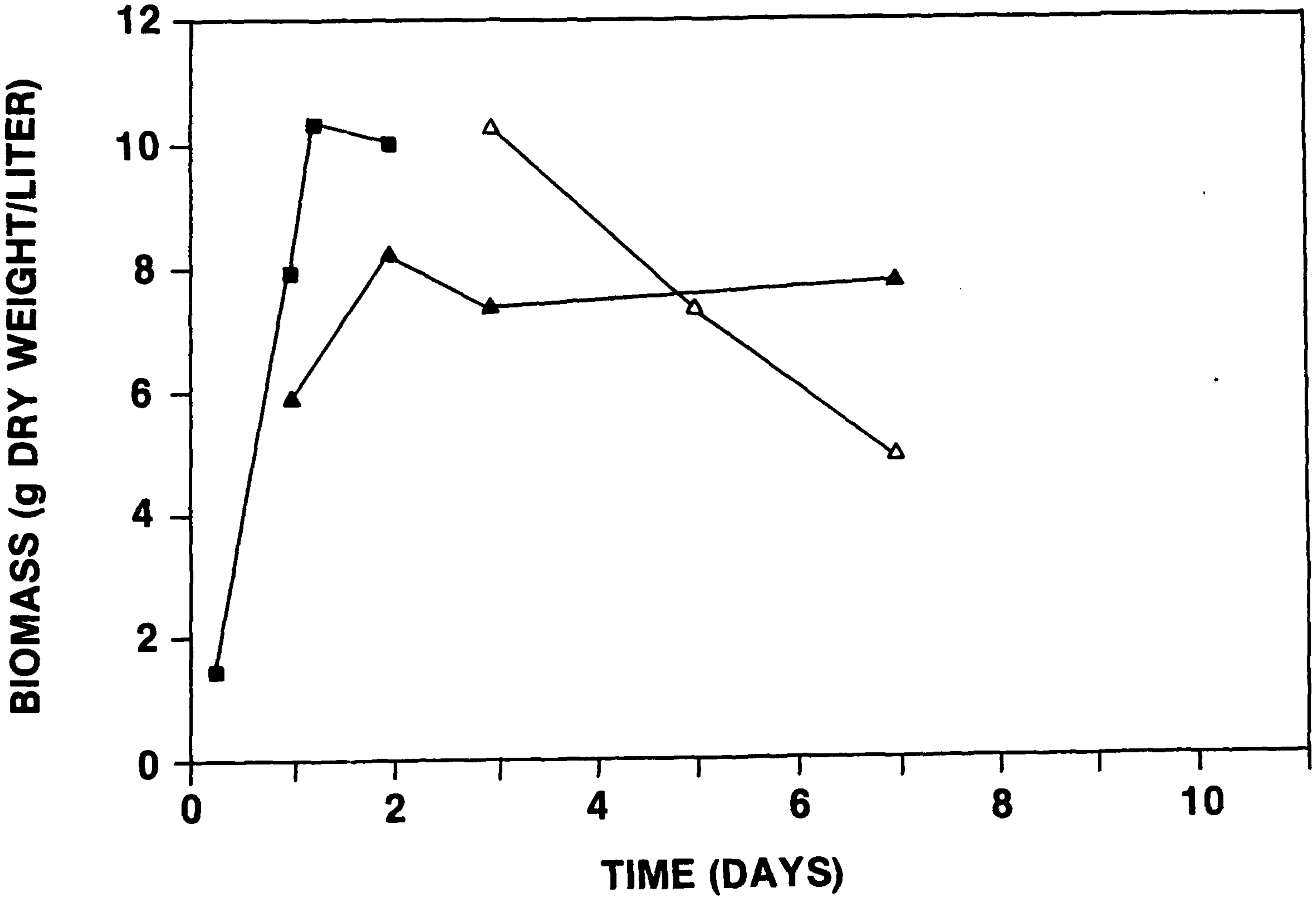

FIGURE 5. BIOMASS PRODUCTION VS. TIME IN COMPLEX MEDIUM, 10 L YPG (1) (■), YPG (2) (▲), PLUG INOCULUM, YPG (△) SPORE INOCULUM

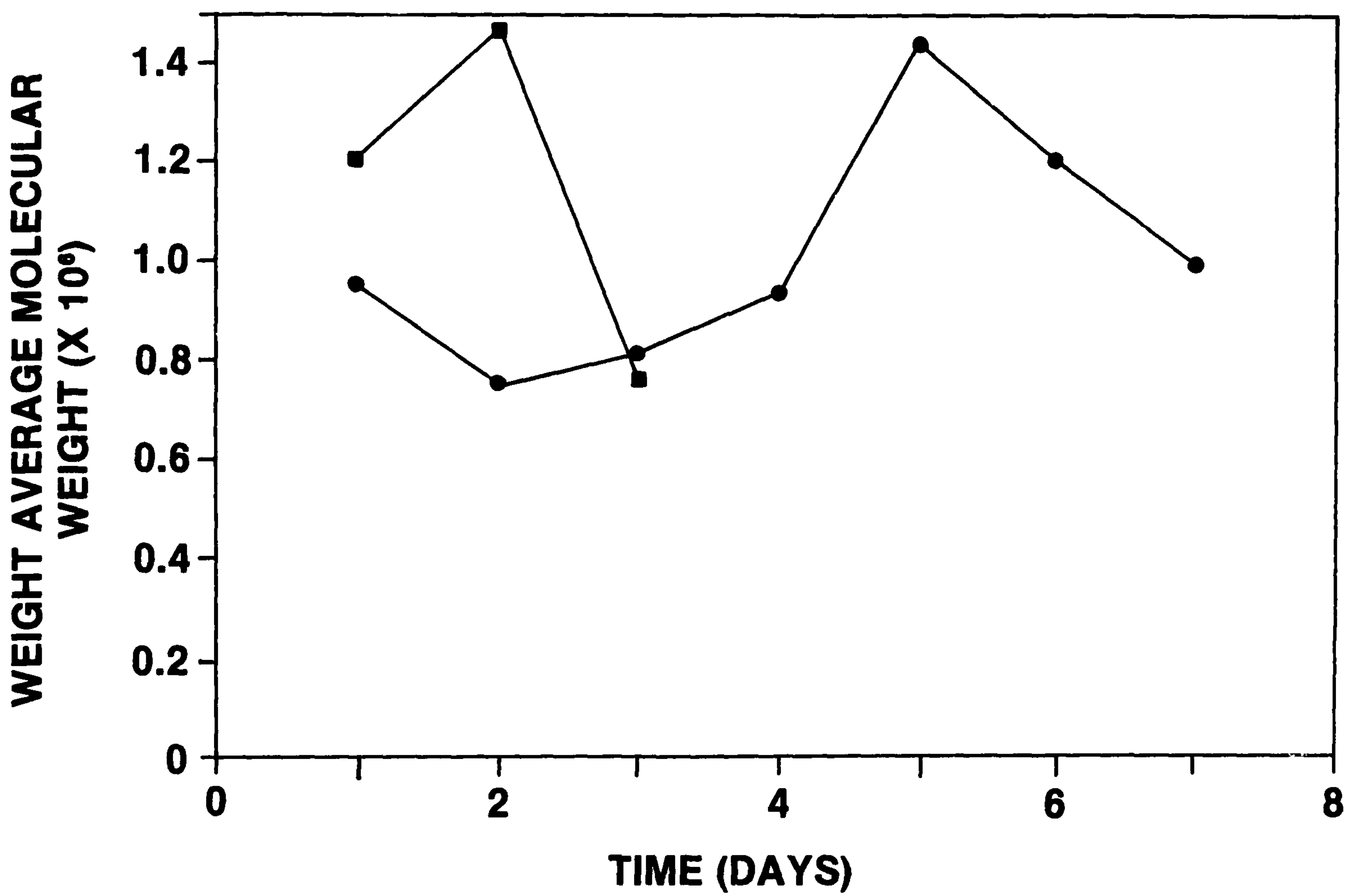

FIGURE 6. WEIGHT AVERAGE MOLECULAR WEIGHT OF CHITOSAN VS. TIME IN DEFINED MEDIUM. FERMENTATION VOLUME OF 750 ml (•), 10 L (▪)

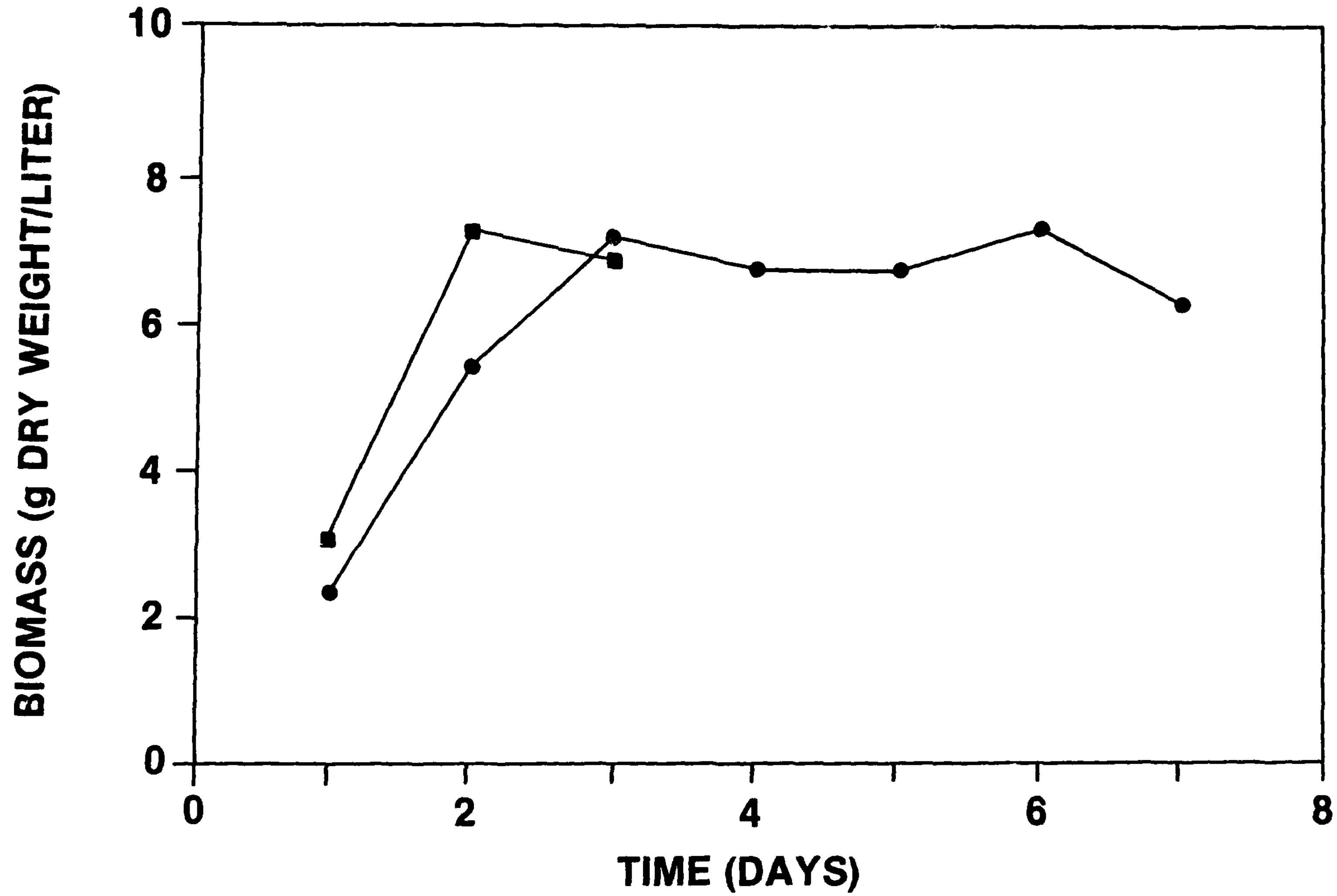

FIGURE 7. BIOMASS PRODUCTION VS. TIME IN DEFINED MEDIUM. FERMENTATION VOLUME OF 750 ml (•), 10 L (■)

which maximum biomass levels were obtained was similar to YPG, the values were 20% to 30% less than those of the complex YPG medium.

The 10-liter batch systems yielded higher weight average MW at an earlier time than in the 750 mL shake cultures (Fig. 6). At two days the weight average MW was 1471 K, whereas the high value of 1442 K did not occur until five days in the 750 mL batch study. Maximum biomass level was obtained by two days at 7.27 g/L, compared to 7.2 g/L at three days for the 750 mL culture (Fig. 7).

pH

In controlled pH experiments, YPG and BG media were adjusted to pH 5 twice a day over a period of 72 hours (Table 1). Cultures without pH adjustment served as controls. Adjustment of the pH of the BG medium resulted in decreased weight average MW and biomass when compared to the unadjusted pH control. There was no significant effect on weight average MW in the YPG medium with pH adjustment in comparison to the control. Biomass decreased from 9.7 g/L in the control to 8.0 g/L in the pH adjusted cultures.

A second set of experiments involved growing *M. rouxii* in small scale defined buffered (TVB-citrate) and complex (YPG) media in which pH was adjusted to a constant value twice daily. The pH values of 3.0, 4.0, 5.0, and 6.0 were evaluated (Fig. 8). The same media, without pH adjustment served as controls. Optimal pH for the production of highest weight average MW was 6.0 for the YPG (521 K) medium and 4.0 for the defined medium (594 K), although differences were small over the pH range studied. The unadjusted YPG control weight average MW was 16% less at 437 K (final pH of 4.2), while in TVB, the weight average MW was 490 K (final pH 5.0). Optimal pH for biomass production was 5.0 in both media, with 9.7 g/L biomass produced in YPG and 6.28 g/L in TVB-citrate. The unadjusted pH YPG control yielded 8.1 g/L and a final pH of 4.6, while the defined medium control resulted in 2.58 g/L at a final pH of 2.3. YPG yielded more biomass over the pH range evaluated. Adjustment of both media to pH 3.0 inhibited fungal growth (Fig. 9).

Inorganic Nitrogen

The complex medium YPG, which contains organic nitrogen in the peptone and to a lesser degree the yeast extract, was supplemented with inorganic nitrogen salts. The addition of $(NH_4)_2SO_4$, $NaNO_2$, or $NaNO_3$ at 0.5% had no significant effect on either weight average MW or biomass (Table 2). All of the weight average MWs were in the 420 K to 500 K range, and yields of biomass were between 9.4 g/L to 10.8 g/L.

Addition of $NaNO_2$ and $NaNO_3$ in place of $(NH_4)_2SO_4$ in the buffered TVB medium was also evaluated. The concentration of

TABLE 1. Effect of pH Adjustment on Molecular Weight Distribution and Biomass in Complex Media

Medium	Biomass* (g/L)	Weight Average Molecular Weight (K)
YPG		
Control	9.7	644
pH Adjust	7.5	702
pH Adjust	8.5	609
Mean pH Adjust	8.0	656
BG		
Control	4.0	643
pH Adjust	3.3	533

* Dry weight

TABLE 2. Effect of the Addition of Inorganic Nitrogen Salts on Molecular Weight Distribution and Biomass in Complex and Defined Media

Medium	Biomass* (g/L)	Weight Average Molecular Weight (K)
Complex (YPG)		
Control	9.62	422
+0.5% NH_4	10.01	471
+0.5% NO_2	9.36	466
+0.5% NO_3	10.79	506
Defined (TVB-Citrate)		
Control	4.79	503
$NaNO_2$	no growth	
$NaNO_3$	7.41	639

*Dry weight

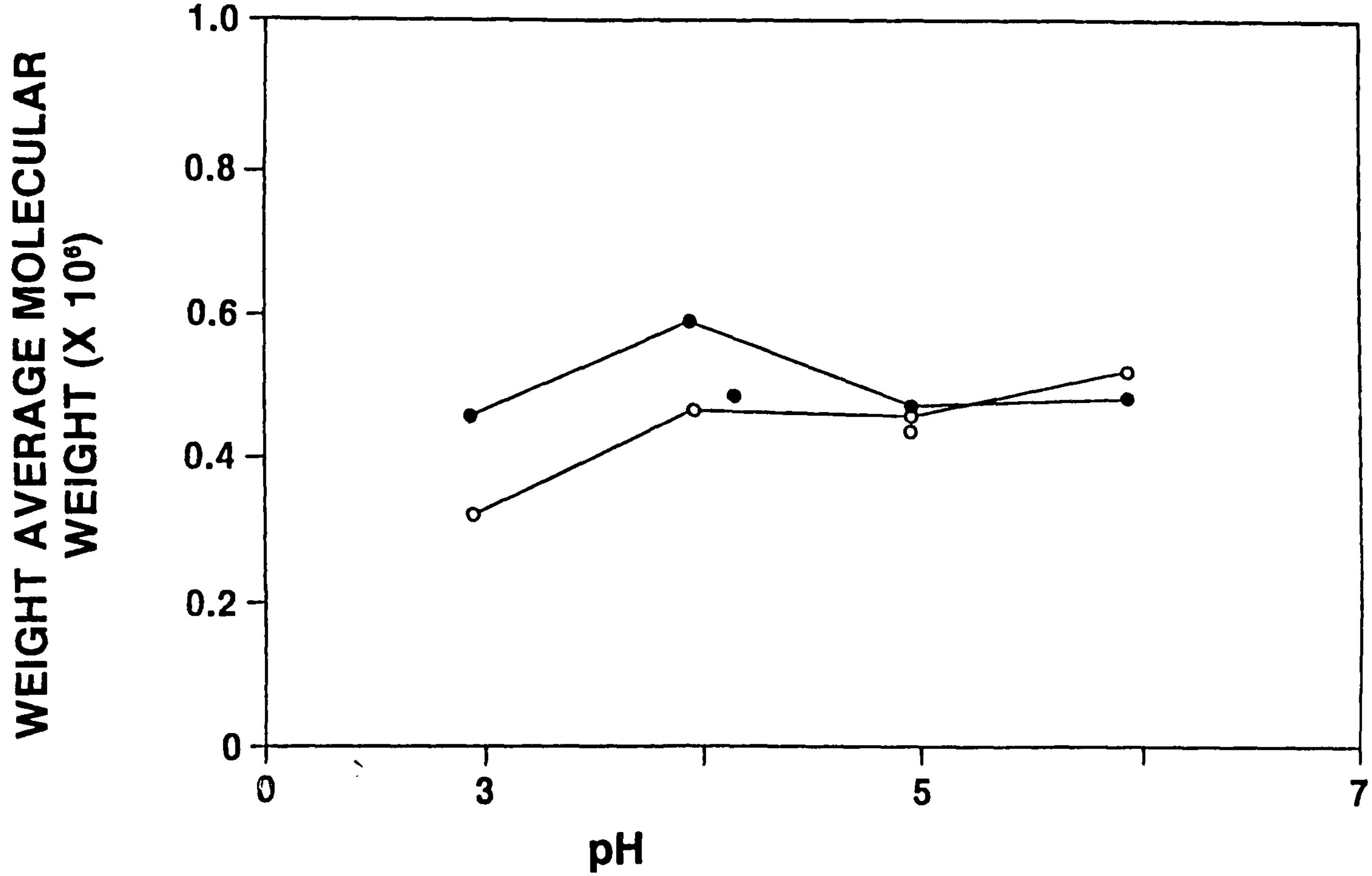

FIGURE 8. EFFECT OF pH ON WEIGHT AVERAGE MOLECULAR WEIGHT IN DEFINED (•) AND COMPLEX (∘) MEDIA. ISOLATED POINTS REPRESENT WEIGHT AVERAGE MOLECULAR WEIGHT WHEN pH IS NOT CONTROLLED (INITIAL pH = 4.2 AND 5.0 FOR DEFINED AND COMPLEX MEDIA, RESPECTIVELY)

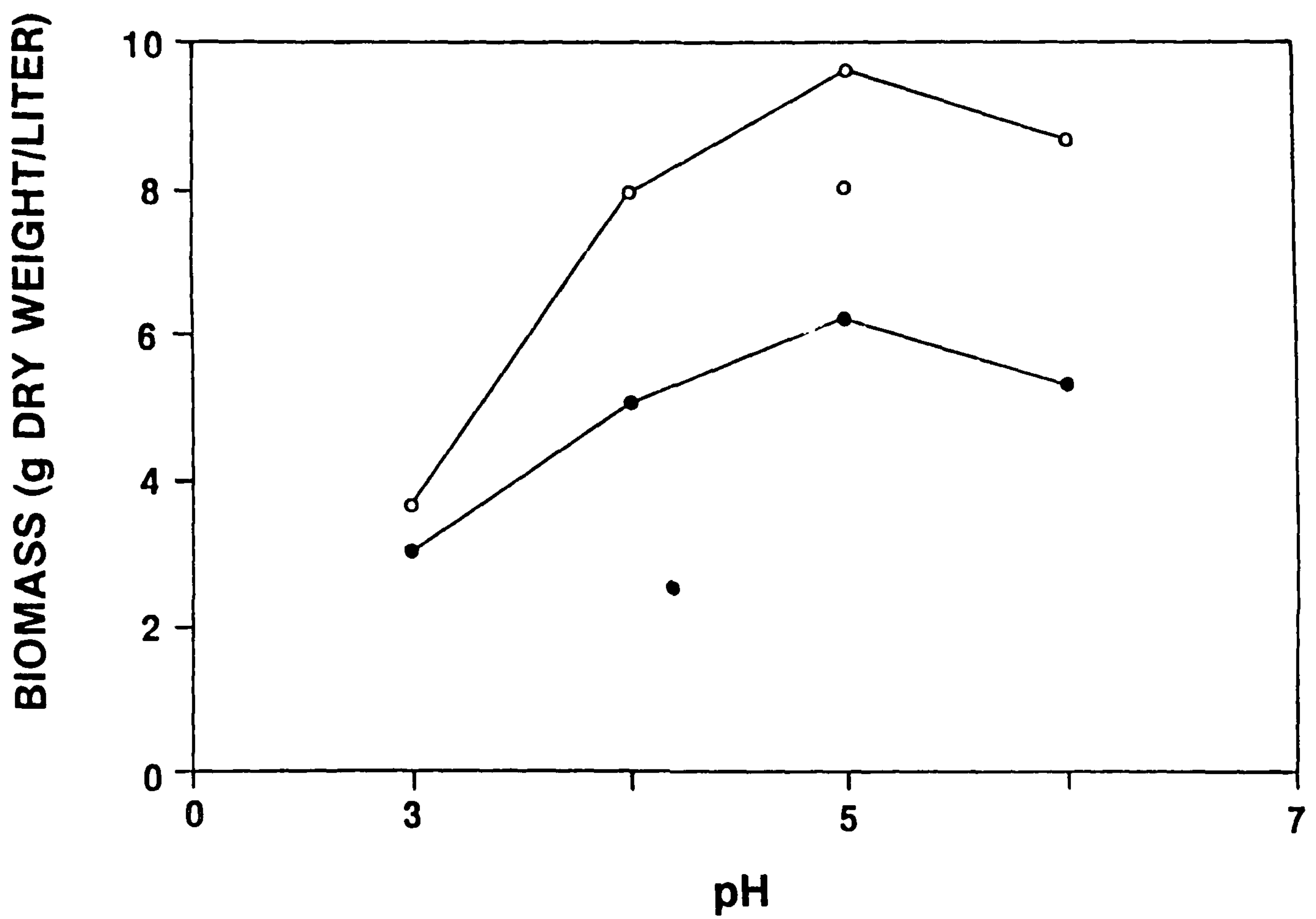

FIGURE 9. EFFECT OF pH ON BIOMASS IN DEFINED (●) AND COMPLEX (○) MEDIA. ISOLATED POINTS REPRESENT BIOMASS WHEN pH IS NOT CONTROLLED (INITIAL pH = 4.2 AND 5.0 FOR DEFINED AND COMPLEX MEDIA, RESPECTIVELY

each inorganic salt was adjusted to the equivalent nitrogen content to that found in $(NH_4)_2SO_4$. The $NaNO_2$ did not support growth. The $NaNO_3$ resulted in higher yields of biomass than the control (7.41 g/L vs. 4.79 g/L) as well as a higher weight average MW (639 K vs. 503 K) (Table 2).

Medium Composition

The three components of the complex medium YPG were evaluated by both eliminating or doubling the concentration of each component (Table 3). The control (YPG, containing 0.3% yeast extract, 1% peptone, and 2% glucose) resulted in 10.3 g/L biomass, while complete removal of peptone or yeast extract resulted in reduced biomass (6.1 g/L and 7.9 g/L, respectively). There was less then 10% change in the biomass when the concentration of any of the components was doubled. The weight average MW was not affected by doubling the glucose concentration to 4% or eliminating the peptone; however, the weight average MW was increased with 2% peptone and with 0.6% yeast extract. In the absence of yeast extract there was an increase in MW distribution.

Trace Elements

The formation of chitosan in the complex media YPG and BG was evaluated in the presence and absence of trace quantities of Fe^{2+} and Mn^{2+} (Table 4). Removal of these trace salts from the BG medium had no effect on weight average MW while biomass was reduced about 25%.

The addition of 0.278 mg/L Fe^{2+} in the form of $FeSO_4 \cdot 7H_2O$ and 2 mg/L Mn^{2+} as $MnCl_2 \cdot 4H_2O$ to YPG provided concentrations comparable to those found in BG. The biomass level with these supplements was 9.2 g/L, compared to 9.6 g/L for the control; the weight average MW was also unaffected. The addition of the trace salts to pH adjusted YPG resulted in no change in either yield or weight average MW.

Biotin was also added to YPG at trace levels (0.1 ng/mL) in an effort to influence morphology (hyphal vs. yeast-like form) and MW distribution. In Candida albicans 0.1 ng/mL biotin has been shown to promote filamentous growth at the expense of the yeast-like form (20). This effect would be desirable in M. rouxii since the hyphal form is easier to lyse than the yeast-like form, and therefore easier to extract the chitosan. In addition, the hyphal form has a higher chitosan content (2). However, no change in morphology was observed when biotin was added. Both the YPG control and biotin-supplemented YPG yielded 9.5 g/L biomass and comparable weight average MWs (Table 4).

Glucose Concentration

The effect of glucose concentration on biomass and weight

cl

TABLE 3. Effect of Concentration of YPG Components on Biomass and Molecular Weight Distribution

Component	Biomass[a] (g/L)	Weight Average Molecular Weight (K)
Peptone (removed)	6.11	364
Yeast Extract (removed)	7.93	456
Control[b]	10.30	327
2% Peptone	10.10	462
4% Glucose	10.70	365
0.6% Yeast Extract	9.50	461

[a]Dry weight
[b]YPG contains 0.3% yeast extract, 1.0% peptone, and 2% glucose.

TABLE 4. Effect of Some Trace Elements on Molecular Weight Distribution and Biomass in Complex Media

Medium	Biomass[a] (g/L)	Weight Average Molecular Weight (K)
YPG		
Control[b]	9.6	556
+ Fe, Mn Salts	8.3	671
+ Fe, Mn Salts	10.1	629
Mean w/Salts	9.2	650
pH Adjust + Salts	9.6	629
+ 0.1 ng/mL Biotin	9.5	640
BG		
Control	4.0	643
- Fe, Mn Salts	3.3	673

[a]Dry weight
[b]Represents average of two samples

average MW was evaluated in YPG and TVB media. The YPG was pH adjusted twice daily to 5.0, while the pH in TVB was controlled with citrate buffer. Weight average MW of chitosan in YPG was not significantly different in 0.5% and 2% glucose; however, 4% glucose yielded 592 K, compared to 473 K and 422 K for 0.5% and 2% glucose, respectively (Fig. 10). In defined TVB, weight average MW increased from 538 K in 0.5% glucose to 821 K in 2% glucose, before declining to 760 K in 4% glucose.

Biomass production (dry cell weight, or DCW) increased with increasing glucose concentration in YPG, ranging from 3.8 g/L DCW in 0.5% glucose to 8.01 g/L in 4% glucose (Fig. 11). In TVB medium, 0.5% glucose yielded 2.39 g/L biomass, rising to a maximum of 5.44 g/L at a glucose level of 2%. Biomass in 4% glucose decreased to 4.1 g/L.

Processing

Type of Acid

White *et al.* (18) recommended the use of 1 N HCl, 10% acetic or 10% formic acid at a ratio of 1:10 (g cell wall:mL acid) for extracting chitosan. It was observed that a larger ratio was required. A 1:10 ratio of HCl resulted in charring of the sample, whereas all the acetic acid was absorbed by the cell wall material. Further study showed that a 1:50 ratio remedied this problem. Acetic acid was the acid of choice because HCl turned the supernatant, and subsequently, the chitosan a dark brown color and little acid insoluble material remained as observed visually.

Acetic Acid

Lyophilized cell wall material was extracted with 5.75% (1 N), 2% and 4% acetic acid for one hour to determined the optimum concentration. Based on the yield of chitosan from cell wall material, 47.5% was obtained with 2% acid, compared to 41.3% yield with 5.75% (1 N) acetic and 33.8% with 4% acetic acid.

For each concentration of acetic acid, the cell wall material was extracted three times for 30 minutes, each time extracting the insoluble material with fresh acid. Chitosan was obtained from each of the first two acid treatments, but the third extraction (total time 90 minutes) produced no chitosan.

Sonification

Mycelia was sonicated both in the presence and absence of Superbrite^{R} Type 119 (approximately 62.5 μm) glass beads (Minnesota Mining & Mfg. Co., Saint Paul, MN). As observed by Lugol's staining, in the absence of glass beads, the mycelia appeared to be completely broken at six minutes, but the arthrospores remained intact. When glass beads were present,

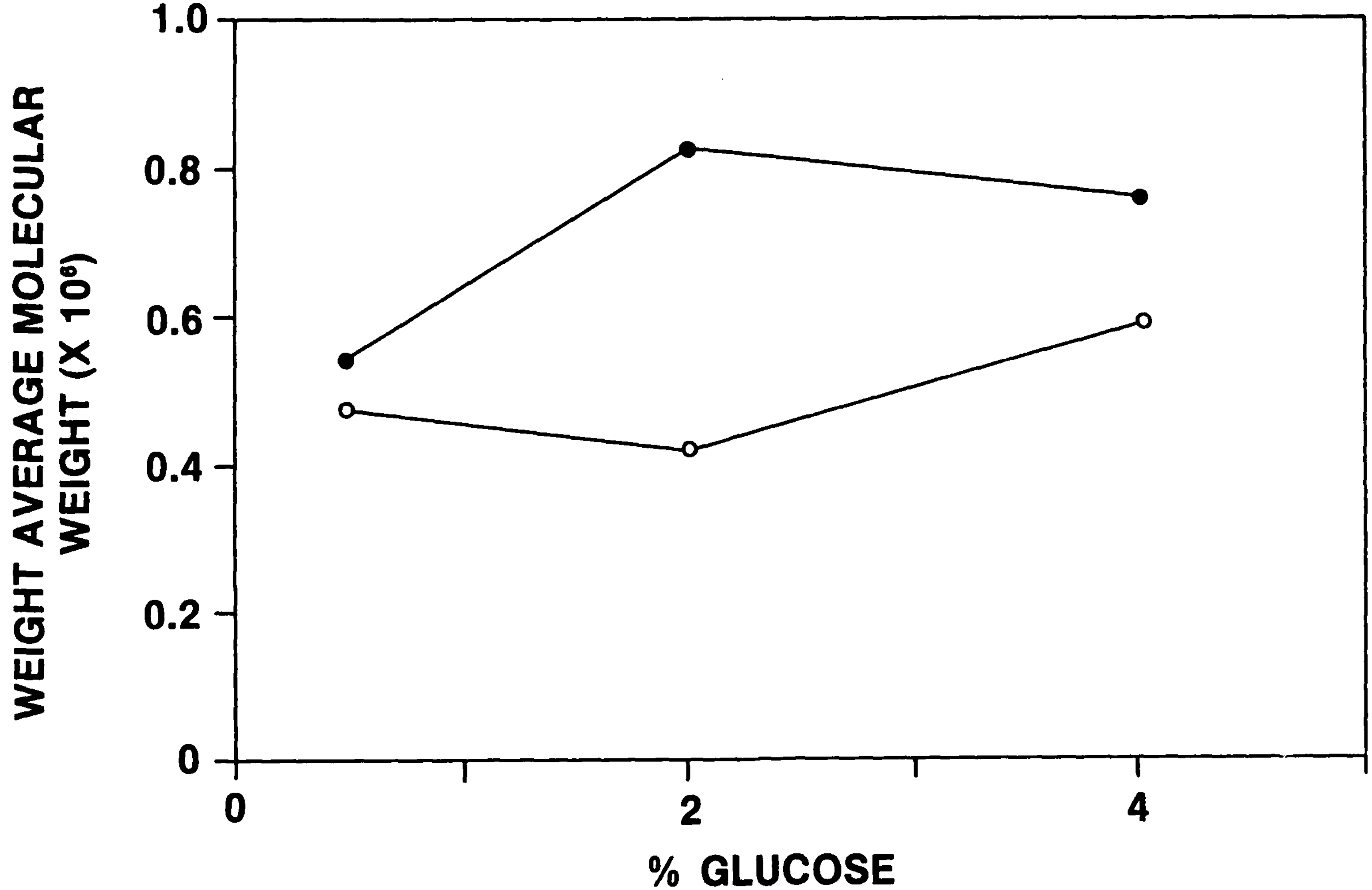

FIGURE 10. EFFECT OF GLUCOSE CONCENTRATION ON WEIGHT AVERAGE MOLECULAR WEIGHT IN DEFINED (•) AND COMPLEX (○) MEDIA, 750 ml

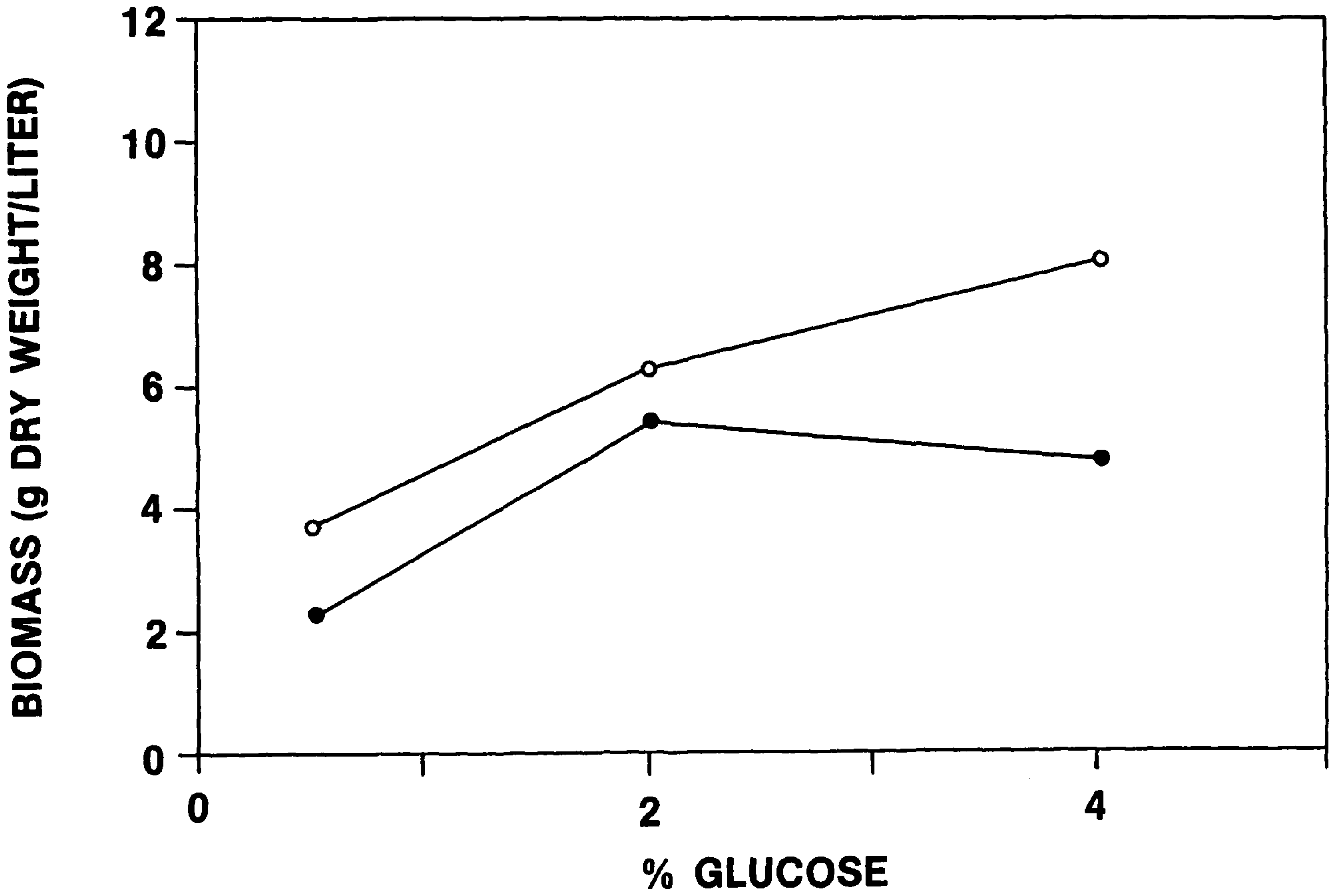

FIGURE 11. EFFECT OF GLUCOSE CONCENTRATION ON BIOMASS IN DEFINED (●) AND COMPLEX (○) MEDIA, 750 ml.

both the mycelia and arthrospore cell walls were broken apart. However, removing the glass beads prior to subsequent processing steps proved to be difficult and therefore was not incorporated into the processing procedure.

Homogenization in Acetic Acid

Cell wall material was homogenized for three minutes in 2% acetic acid at 1:100 ratio (g cell wall/mL acid) in a Waring blender prior to 2 hour refluxing to increase the surface area accessible to the acid. Evaluation of this parameter was based on the percent yield of chitosan extracted from the cell wall. The control, not homogenized, yielded 32.7% chitosan (7.2% of total biomass), while the cell wall homogenized in acetic acid yielded 43% chitosan (9.5% of biomass).

Homogenization of cell wall material isolated from mycelia sonified for four minutes yielded 41.0% chitosan (9.1% of biomass), while the control, sonified four minutes but not homogenized produced 30.0% chitosan (6.6% of biomass).

Pretreatment

Mycelia were pretreated by soaking in water for seven days, or by boiling for 30 minutes in water. Observation of mycelia made with Lugol's stain demonstrated that neither treatment was effective in breaking apart the arthrospores.

Sodium Hydroxide

White (18) used an extraction method which treated the wet mycelium with 1 N NaOH at a ratio of 1:40 (g wet biomass:mL NaOH). A NaOH concentration of 1:20 ratio was compared to 1:40 to observe differences in MW distribution. In three of four samples tested (Table 5), there was no significant difference in MW distribution at a 1:20 ratio vs. the 1:40 ratio.

Storage

Refrigeration ($4^{o}C$) of the harvested mycelia was studied to determine if there was any change in MW distribution. Two samples were evaluated (Table 6) one stored for 11 days (MW distribution of 1093 K), compared to a one day control (934 K). 18 day refrigeration resulted in 1022 K MW, while a second control (one day) yielded 1138 K.

Acid Extraction

White '18) used refluxing with the following acids to extract chitosan from NaOH treated mycelia: 1 N hydrochloric, 10% formic or 10% acetic. The ratio of cell wall material to acid is 1:10 (w/v), and the time of treatment 3, 6, 12, and 24 hours.

TABLE 5. Effect of Ratio of Wet Biomass (g) to 1 N NaOH on Weight Average Molecular Weight*

Medium	Incubation (hr)	Length of Refrigeration (days)	Ratio Biomass:NaOH	Weight Average Molecular Weight (K)
TVB/buffer	93	11	1:40	1093
			1:20	904
TVB/buffer	72	14	1:40	1057
			1:20	959
TVB/buffer	72	3	1:40	771
			1:20	1089
TVB	72	0	1:40	883
			1:20	877

*At a ratio of 1:40, the weight average MW (mean of four samples) was 951 K. The weight average MW (mean of four samples) at a 1:20 ratio was 957 K.

TABLE 6. Effect of Length of Refrigeration (4°C) on Molecular Weight Distribution

Sample	Length of Storage(Days)	Weight Average Molecular Weight (K)
1	1	934
	11	1093
2	1	1138
	18	1022

Soxhlet extraction was evaluated for this purpose (Table 7). Refluxing previously frozen mycelia in 2% acetic acid yielded 662 K weight average MW; the use of a cellulose thimble (Whatman LabSales, Inc., Hillsboro, OR) during Soxhlet extraction with 2% acetic acid resulted in 303 K. Acetic acid concentration was increased to 5%, and both cellulose and Teflon (Chemplast, Inc., Wayne, NJ) extraction thimbles were evaluated. Weight average MW of 343 K and 430 K were obtained with the cellulose and Teflon thimbles, respectively. While these values are higher than cellulose in 2% acetic acid, they are still far below the weight average MW observed during refluxing.

The temperature of 5% acetic acid during refluxing was 100°C to 105°C, while liquid within the Soxhlet extraction thimble reached 70°C to 75°C. Improved insulation of the apparatus raised the temperature to 90°C to 95°C. Using previously refrigerated mycelium as controls (Table 8), refluxing in 2% and 5% acetic acid yielded MW values of 341 K and 411 K, respectively. By lowering the temperature of the refluxing solution to approximately 75°C, a MW distribution of 392 K is achieved. Soxhlet extraction, however, yielded yet lower values. Extraction with a cellulose thimble and 5% acetic acid resulted in 213 K weight average MW at 60°C, and a MW distribution of 243 K using the foil-covered apparatus at 95°C.

Lyophilization

The necessity of lyophilization of NaOH treated cell wall material (18) was evaluated on mycelia previously stored at different temperatures. At room temperature, MW distributions were similar with lyophilization resulting in 528 K; without lyophilization resulting in 548 K (Table 9). For mycelia stored at 4°C, the nonlyophilized weight average MW was 557 K, compared to 482 K in the lyophilized material. Differences were greater in previously frozen material, where nonlyophilized material yielded 880 K and the lyophilized 774 K.

Derivatization of Chitosan

Treatment of chitosan with methanesulfonic acid and phosphorus pentoxide produced a water-soluble chitosan derivative. However, of the 0.5 grams of chitosan treated, only 0.084 g or 16.8% was recovered. In comparison, 50% to 80% of chitin derivatized as above was recovered. The weight average MW of the chitosan was 593 K initially, but decreased to 27.8 K after treatment. Viscosity decreased 40 minutes after the phosphorus pentoxide was added to the chitosan-methanesulfonic acid solution. The loss of viscosity may have been caused by degradation of the polymer by the methanesulfonic acid.

TABLE 7. Effect of Refluxing vs. Soxhlet Extraction of Chitosan from Cell Wall Material

Treatment	Extraction Thimble Composition	% Acetic Acid	Weight Average Molecular Weight (K)
Reflux	-	2	662
Soxhlet	Cellulose	2	303
Soxhlet	Cellulose	5	343
Soxhlet	Teflon	5	430

TABLE 8. Effect of Temperature on Refluxing vs Soxhlet Extraction of Chitosan from Cell Wall Material

Treatment*	Temperature (^{o}C)	Weight Average Molecular Weight (K)
Reflux	100	411
Reflux	75	392
Soxhlet	60	213
Soxhlet	95	243

*5% acetic acid

TABLE 9. Effect of Cell Wall Lyophilization on Molecular Weight Distributions for Mycelium Stored at Different Temperatures

Mycelium Storage Temperature (°C)	Weight Average Molecular Weight (K) Nonlyophilized	Lyophilized
25	548	528
4	557	482
-18	880	774

TABLE 10. The Effect of Centrifugation or Filtration of Chitosan Samples on Molecular Weight Determination

	Weight Average Molecular Weight (K)			
Treatment / Sample	1	2	3	Mean
Control	909	914	1016	946
Centrifugation (3000 rpm)	671	745	724	713
Filtration (0.5 um)	630	nd*	693	662

*not determined

Sample Preparation

Removal of insoluble material from chitosan samples prevented pressure build up on the GPC during MW determinations. However, it was observed that weight average MW values in certain samples changed after centrifugation or filtration. As an example, insoluble material was removed by centrifugation (3000 rpm for five minutes) or filtration through a 0.5 μm scintered stainless steel filter (Table 10). The unclarified controls produced a mean weight average MW of 946 K (N=3), compared to 713 K (N=3) after centrifugation and 662 K (N=2) in filtered samples. It has been reported that filtration may lead to shearing of high MW polymers, reducing the overall weight average MW. The insoluble material appeared to be partially solubilized material, possibly of high MW. This fraction was pelleted during centrifugation and was not injected onto the GPC for analysis. Once these problems associated with centrifugation and filtration were identified, samples were not routinely clarified prior to injection on the GPC.

The presence of insoluble material in the injected sample resulted in an increase in GPC system pressure and increased pressure within the GPC system appeared to affect the determination of weight average MW (Table 11). The increase in pressure appeared to correlate with increasing calculated weight average MW values. A sample containing insoluble material was analyzed with sequential injections. There is a small increase in pressure for injections 1 through 3. Weight average MW increased to 331 K and 335 K, respectively; with subsequent injections, the system pressure increased above 22 bars. After replacement of the 0.5 μm pre-column filter, pressure was restored to the normal operating range of 16 to 18 bars, and the next injection yielded a weight average MW of 241 K.

Other sample treatments were evaluated for removal of insoluble material. Dilution of sample concentration from 0.1% to 0.05% (w/v) resulted in an increased weight average MW from 168 K to 211 K. Solubilization of the same chitosan sample at an elevated temperature of 30°C to 35°C also resulted in an increased MW to 214 K; however, storage of chitosan samples at 30°C to 35°C led to a decline in MW from 162 K to 104 K after 96 hours. Solubilization of chitosan in mobile phase containing 5% acetic acid appeared to decrease the amount of insoluble material as well as increase weight average MW. This preliminary evaluation of final chitosan samples preparation suggested that the insoluble material may be a high MW fraction, or possibly more highly acetylated; the material becomes more like chitin as acetylation increases.

Analysis

Infrared spectrophotometry was the first method to be evaluated to determine the degree of acetylation of commercial

TABLE 11. Effect of GPC Pressure on Weight Average Molecular Weight

Sample	Pressure (bars)	Weight Average Molecular Weight (K)
1	16-19	228
2	20	235
3	20-22	239
4	23-25	331
5	27-30	335
after replacement of pre-column filter:		
6	16-18	241

and fungal chitosan. This procedure proved unreliable due to difficulty in calibrating the high acetyl content range, as well as high variability between samples from the same source. Two other methods, titration and first derivative ultraviolet spectrophotometry yielded percent deacetylation values found in Table 12. The majority of commercial and fungal samples exhibit greater than 70% deacetylation. However, wide variations within samples occur with the titration method, indicated by the large standard deviations.

Values determined by titration are at best an approximation. In an effort to determine variability within samples, multiple aliquots of chitosan samples were analyzed in series on the GPC (Table 13). The standard deviations as a percentage of the mean were in the range of 1.8% to 5.4% for four of the samples, while the remaining samples (2 and 3 in Table 13) had higher percent standard deviations, 11.8% and 10.8%, respectively.

Variability between chitosan samples isolated from different cultures of *M. rouxii* grown under identical culture conditions was also determined (Table 14). Under standard conditions of 750 mL culture of YPG medium, grown for three days at 125 rpm, the mean weight average MW was 513 K $\pm$ 85 K (N=5). Biomass levels ranged 8.10 g/L to 10.3 g/L DCW. In pH adjusted YPG cultures, the mean weight average MW was 594 K $\pm$ 274 K, with a biomass range of 5.25 g/L to 10.3 g/L DCW. The biomass in the defined TVB-citrate media ranged between 4.1 g/L to 7.2 g/L DCW. The mean weight average MW for this culture was 658 K $\pm$ 167 K. Since the range of weight average MWs was large, it was difficult to compare MW values obtained from different experiments. At least two reasons for the ranges can be identified. As discussed below, samples act differently on GPC columns of different ages despite calibration, which may contribute to the difference in MW distributions. More importantly, as illustrated by the biomass data (Table 14), inoculation of liquid cultures by agar plugs did not lead to consistant biomass levels. It was felt that less variability between cultures could be obtained using a standardized spore inoculum, although no data were generated concerning this issue.

Some samples were analyzed several times for weight average MWs over an extended period of time. It was difficult to obtain consistent results when several months passed between determinations, column age being a significant factor (Table 15). In general, the tendancy was for weight average MW values to decline as the GPC columns aged. This occurred despite the fact that the columns were re-calibrated. During each injection a small amount of cationic chitosan may bind irreversibly to the column packing, possessing a slightly negative charge. As this material accumulated, the elution time and therefore MW distributions of the chitosan changed. The pullulan standards were not reactive with the column packing and re-clibration did not completely reflect the column condition with regard to chitosan.

TABLE 12. Degree of Deacetylation of Commercial and Fungal Chitosan Determined by Various Methods

Sample	Percent Deacetylation ±Standard Deviation	
	Titration	First Derivative Ultraviolet Spectrophotometry
Commercial		
Sigma (114F-0141)	84.5±25.3	79.6±0.45
Sigma (47F-0226)	nd*	84.7±1.8
Protan (Seacure)	nd	80.5±1.8
Fungal (*M. rouxii*)		
(63)TVB 0.5 L/hr	85.0±18.2	91.0±0.32
(33)YPG pH 5	91.4±2.6	88.6±1.1
(37)YPG/biotin	75.8±10.3	nd
(75a)TVB-c 0.5 L/hr	60.3±4.9	nd
(85)TVB-c 48 hr 1:20	72.6±1.8	nd
(85)TVB-c 48 hr 1:40	70.8±0.71	nd
(31)YPG pH 3	84.0±11.3	nd
(47)YPG 144 hr	84.5±1.3	nd
(49)YPG 72 hr	nd	87.1±1.4
(83)TVB-c 72 hr 1:20	nd	91.5±0.7
(83)TVB-c 72 hr 1:40	nd	88.2±2.9
Glucosamine (standard)	105±7.0	nd

*not determined

TABLE 13. Variability of Weight Average Molecular Weight within a Chitosan Sample

Sample	Aliquot	Weight Average Molecular Weight (K)	Mean (± Standard deviation)
1	a	913	925 ± 30
	b	959	
	c	903	
2	a	717	817 ± 96
	b	909	
	c	825	
3	a	202	227 ± 24.5
	b	229	
	c	251	
4	a	464	474 ± 8.4
	b	473	
	c	480	
	d	469	
	e	485	
5	a	277	269 ± 14.5
	b	257	
	c	273	
	d	286	
	e	251	
6	a	228	234 ± 5.6
	b	235	
	c	239	
	d	241	

TABLE 14. Variability of Biomass and Weight Average MW of Chitosan Isolated from *M. rouxii* Grown Under Identical Culture Conditions

Culture Condition	Sample #	Biomass[a] (g/L)	Weight Average Molecular Weight (K)	Mean (± standard deviation)
750 mL YPG, 72 hour, 125 rpm	1	10.30	538	513±85
	2	9.49	644	
	3	9.46	508	
	4	10.10	440	
	5	8.10	437	
750 mL YPG, 72 hour, 125 rpm pH adjusted	1	7.28	702	594±274
	2	8.32	609	
	3	10.30	260[b]	
	4	9.70	460	
	5	8.40	1123	
	6	6.30	422	
	7	5.25	584	
750 mL, TVB-citrate 72 hour, 125 rpm	1	7.20	821	658±167
	2	6.90	457	
	3	4.16	664	
	4	5.44	821	
	5	4.10	526	

[a]Dry weight

[b]N=7

TABLE 15. Effect of Length of GPC Column Use on Molecular Weight Determinations

Sample	Length of Time of Column Use (months)	Weight Average Molecular Weight (K)
1	2	946[a]
	6	251[b]
2	2	574
	9	335
3	5	407
	10	800[c]
4	4	1089
	8	659

[a] N=3

[b] N=4

[c] N=2

It has been recommended that a mobile phase of increased ionic strength would minimize cationic polymer interaction with the column packing (11). This recommendation resulted from work with glycol chitosan, which is water soluble. Wit 0.1 M sodium acetate in the mobile phase, the weight average MW of commercial chitosan had a mean of 1174 K (N=5). When sodium acetate in the mobile phase was increased to 0.3 M, the mean weight average MW declined to 700 K (N=4). A change of conformation in the polymer may occur with the increased ionic strength which in turn would change in MW value.

Based on the above evaluation of many different sample preparation procedures, we concluded that comparisons of results from different studies carried out at very different stages of column age/use were not feasible, unless improved column packing could be found that does not react with the chitosan. Comparisons from studies and samples run at similar times are valid.

DISCUSSION

A number of growth and processing conditions influenced biomass production and the weight average MW of chitosan. Growth conditions included length of incubation, culture vessel volume, the type of inorganic nitrogen salt in defined medium, and medium component concentration. The processing variables affecting the yield of chitosan extracted were the type and strength of acid, and homogenization of cell wall material in acid before refluxing.

In complex media, weight average MW distribution increased rapidly over three days, then declined as length of incubation increased. This was especially true in the two separate growth studies using the YPG medium. The use of plug inocula may have resulted in different growth rates between cultures, and would be reflected in the difference of MW values. The decline of MWs with time may indicate that the chitosan is modified, degraded or turned over in the cell wall, making extraction more difficult. There is documentation of the production of chitosanases in a strain of *M. rouxii* (13), which undergoes autolysis. In the strain of *Mucor* used in the study, autolysis did not begin until seven days (14). We observed a decline in weight average MW prior to seven days. It is believed that chitosanases were not responsible. The chitosan must become accessible for the enzyme to be functional. Chitosan does not appear to become increasingly accessible, as the amount of chitosan extracted from the cell wall does not increase with time of incubation.

The difficulty in extracting high weight average MW chitosan may be due to the formation of an increased number of arthrospores with time as environmental stress occurs (2). As was observed by Lugol's stain, the arthrospores proved to be more difficult to break apart during the extraction procedure, resulting in the chitosan being more difficult to extract. In addition, in the early stages of arthrospore production, the

hyphal cell wall continues to surround the arthrospore cell wall (1). In mature arthrospores, the hyphal cell wall becomes degraded. There appears to be no data on the chitosan of arthrospores, but it may be of a different nature then hyphal chitosan, or less accessible to disruption and extraction from the cell wall.

Culture volume had an effect on the rate of growth of the fungus, which influenced the yield of biomass and the MW distribution. Mucor grown in large-scale, 10-liter batch cultures grew more rapidly than in the 750 mL cultures. This may be due to better mixing of the growth medium by the impellers, which facillitates more efficient transfer of nutrients and dissolved oxygen to the growing organism. Increased availability of dissolved oxygen would also counteract the effects of carbon dioxide in the medium, which promoted the formation of the yeast-like rather than the hyphal form of the fungus (2).

The concentration of YPG medium components also exhibited an effect on MW distribution as well as biomass production. Elimination of either yeast extract or peptone from the YPG medium significantly reduced biomass levels, indicating the need for the organic nitrogen or amino acids found in these components. Doubling the concentration of yeast extract or peptone increased weight average MW, as did the absence of yeast extract. It appears therefore that a component of the peptone, which consists primarily of nitrogen and minerals, appears to affect weight average MW.

Processing steps for the isolation and purification of chitosan are important in terms of the yields and MW distribution of the polymer. The type of acid used for extraction was important. White et al. (18) determined that 1 N (8.3%) hydrochloric acid was the acid of choice; however, this decision was based on yield at the expense of degree of acetylation. There were no MW determinations made. Hydrochloric acid was initially used but discontinued when the cell wall material turned brown and appeared to be degraded during the extraction.

Acetic acid at different concentrations was evaluated. There were differences in the extraction efficiency, with 2% acetic acid producing the highest yield when compared to 1 N and 4%. It may be possible that the 1 N (5.75%) and 4% acetic acid treatments were too strong and hydrolysis of the chitosan occurred. Homogenization of the cell wall prior to refluxing in acetic acid increased the efficiency of chitosan extraction, probably due to increased surface area of the cell wall material.

A number of growth conditions were evaluated that did not have an effect on the yield of biomass or MW distributions of the chitosan including adjustment of culture media to a constant pH, addition of inorganic nitrogen salts to YPG, and supplementation of YPG with trace quantities of iron, manganese, or biotin.

Adjusting the pH (3.0, 4.0, 5.0, and 6.0) in defined and complex media to a constant value did not appear to affect weight average MW of the chitosan. Biomass was not affected over the

range pH 4.0 to 6.0; however, at pH 3.0 reduced biomass was observed. However, the pH of a complex medium of a differant formulation was found to have an effect on the production of biomass (8).

Iron and manganese salts were added to the YPG medium in an attempt to influence the MW distributions of the chitosan. These salts were present in BG, but thought not to be present in sufficient quantities in YPG. Early studies showed that MW distributions of the chitosan from fungus grown in BG were higher than those from YPG. The two enzymes responsible for the organism's chitosan biosynthesis, chitin synthetase and chitin deacetylase, require Mn^{2+} for functionality (4). Later it was learned that the peptone and yeast extract present in YPG should have sufficient quantities of these elements. Therefore, addition of these salts to YPG was unnecessary and did not influence weight average MW. Biotin was added to YPG as a growth factor. In Hyphomycetes biotin has been shown to promote hyphal growth at the expense of the yeast-like form (20). This was desirable in *M. rouxii* since the hyphal form was easier to break apart during the extraction process than the yeast-like form. However, despite the addition of biotin, the desired effect on morphology was not observed. Perhaps a component of the complex medium prevents this change of morphology from taking place.

Addition of inorganic nitrogen salts to YPG did not affect biomass or chitosan MW distributions, presumably because the organic nitrogen present in the peptone and yeast extract was preferentially utilized. In the buffered defined medium TVB, $NaNO_3$ increased biomass and weight average MW when used in place of $(NH_4)_2SO_4$. $NaNO_2$ did not support growth.

Several processing parameters evaluated had no effect on the weight average MW or the amount of chitosan extracted from the cell wall. Sonification and pretreatment of mycelia by soaking or boiling in water produced no difference in polymer MW distribution. The use of Soxhlet extraction in acetic acid in place of refluxing was attempted in order to produce an end-product free of insoluble material. The final product had little to no insoluble material, but the weight average MW was reduced (Table 7). The porosity of the extraction thimbles, 10-15 μm for cellulose, 50 μm for Teflon$^{(R)}$, may have prevented the recovery of the high MW fraction.

Other steps in White's, *et al.* (18) extraction procedure were modified or eliminated to improve the efficiency of the isolation procedure. The criteria for changes made in this procedure were that modifications could not reduce weight average MW or the amount of chitosan extracted. Immediate processing of mycelia after harvesting was not always possible, and storage of the mycelia was found to be acceptable. Lyophilization of the cell wall material was eliminated, thereby significantly decreasing the total processing time. The ratio of cell wall material to 1 N NaOH (w/v) was reduced from 1:40 to 1:20,

reducing the volume of alkali. These modifications resulted in shorter processing time and reagent requirement reduction, without a loss in chitosan weight average MW.

Three methods to determine percent deacetylation were examined; infrared spectrophotometry, titration, and first derivative ultraviolet spectrophotometry. Standardization of the IR spectrophotomter with solutions of known percent deacetylation was not possible and the method abandoned. Titration deacetylation values were variable within a given sample, possibly due to solution viscosity, foaming, and precipitate formation during titration. Values determined by first derivative UV spectrophotometry were more consistant for a given sample, as shown by small standard deviation. However, some aspects cited in the original method could not be duplicated. Peak absorbance of the N-acetyl glucosamine standards was not always at 199 nm; peak wavelength steadily increased as N-acetyl glucosamine concentration increased. Measurements at 202 nm (zero order crossing point) were not independent of acetic acid concentration, as Muzzarelli claimed. An increase of acetic acid caused a reduction of peak height and an increase of peak wavelength in samples and standards. These events raise possible questions about the accuracy of the method.

There is room for additional improvement of the extraction and purification procedure, especially in terms of decreasing the insoluble material that is present in some samples. In addition, obtaining reliable MW distributions over a period of time will remain a problem until an improved column packing can be found that does not react with chitosan. The need for standardized culture inoculum was evident, as biomass levels and MW distributions were variable between cultures grown under the same conditions.

CONCLUSIONS

Culture conditions for the fungus *Mucor rouxii* and processing steps critical in the isolation and purification of chitosan from the fungal cell wall were studied. This represents the first such study where these factors were correlated to the yield and MW distribution of the chitosan. Based on the results of these studies, length of incubation, culture volume, inorganic nitrogen source in defined medium, and medium component concentration in complex medium were important factors that influenced polymer MW distribution, while addition of trace elements and glucose concentration were some of the factors that did not appear to influence polymer MW. The type and strength of acid, as well as homogenization of cell wall material prior to acid extraction were important processing steps affecting the amount of chitosan extracted. Qualitative values for the degree of deactylation can be determined by the methods evaluated.

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